IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants: Yukoh HIEI et al

Serial No.: 10/089,695 Group: 1638

Filed: May 21,2002 Examiner: Worley

For: METHOD FOR PRODUCING EFFICIENTY OF GENE TRANSFER INTO PLANT CELLS

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C., 20231

Sir:

I, Yukoh HIEI, a nation of Japan, residing at c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Iwata-shi, Shizuoka 438-0802, Japan, do hereby declare as follows:

I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Office Action dated April 25, 2008, in which claims 1, 3-7, 12, 14-15, 17,18, 20,21, 23 and 24 are rejected.

To show the patentability of the present invention, I carried out the experiments described below.

EXPERIMENT 1

Enhancement of *Agrobacterium*-mediated gene transfer in rice immature embryos pre-treated with centrifugation at 1,000 xg and 20,000 xg for very short duration.

Materials and Methods

(1) *Agrobacterium* Strain and Plasmid

As the *Agrobacterium* and its vector, LBA4404(pSB134) (Hiei and Komari, 2006) was used. The T-DNA region of pSB134 has a hygromycin-resistant gene (*hpt*) regulated by maize ubiquitin promoter and a GUS gene regulated by the 35S promoter of CaMV and having the first intron of the catalase gene of castor-oil plant.

(2) Sample Varieties and Tissue

As the sample variety, Yukihikari, which is the variety of Japonica rice, was used. As the sample tissue, immature embryo was used. The preparation method of the tissue is the same as that described in the specification of the present patent application.

(3) Centrifugation Treatment

Rice immature embryo was placed in a 1.5 ml centrifugal tube containing 1 ml of sterilized water. The tube was subjected to centrifugation treatment for 1 second, 10 seconds or 60 seconds at 1,000 xg, or 1 second at 20,000 xg. In addition to the experimental plots with these accelerations, an experimental plot with no centrifugation was added, thus, five experimental plots were prepared in total. In each experimental plot, 15 immature embryos were used. After the centrifugation, the immature embryos were infected with *Agrobacterium*.

(4) Infection of *Agrobacterium* and Co-culturing

The method of infection of the immature embryos with *Agrobacterium*, the method of co-culturing and the method of GUS assay of the immature embryos after the co-culturing were the same as described in specification of the present patent application. In the present test, the GUS expression levels in the immature embryos were expressed in values as GUS Activity Index as follows: Each of the immature embryos was then visually examined for the percentage of the sum of the blue areas to the total surface area of the scutellum. A score was given according to the percentage; score 0.0 was given for 0%, score 0.5 for between 0% and 1%, score 5.5 for between 1% and 10%, score 17.5 for between 10% and 25%, score 37.5 for between 25% and 50%, score 62.5 for between 50% and 75%, and score 87.5 for 75% and 100%. The average of the scores in an

experimental plot was recorded as the GUS Activity Index. The co-culturing was carried out for 6 days. The experiment was carried out two times.

Results and Discussion

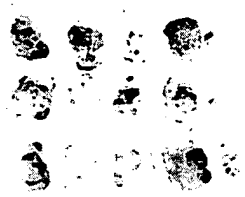
A tendency was observed that the growth of the hypocotyl is inhibited and the scutellum is grown during the co-culturing in the immature embryos subjected to centrifugation. The state of GUS expression in the immature embryos after the co-culturing is shown in Figure 1 and 2. The percentage of the area in the scutellum, which showed GUS expression was apparently increased by the centrifugation treatment when compared with the non-treated group (Figures 1 and 2, and Table 1). In cases where the centrifugation at 1,000 xg was performed for 1 second, 10 seconds or 60 seconds, the GUS-expressed area was not so different among the groups (Figure 1 and 2, and Table 1). In contrast, in the group subjected to 20,000 xg for 1 second, the percentage of the GUS-expressed area was considerably increased when compared with the cases in the groups subjected to 1,000 xg (Figures 1 and 2, and Table 1). Thus, the centrifugation treatment at 1,000 xg or more has an effect to prominently increase the gene transfer efficiency even if it is performed for very short duration such as 1 second.

Cited Reference

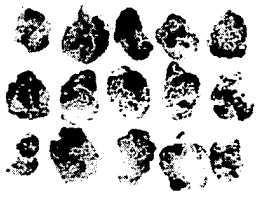
Hiei Y, Komari T (2006) Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*. Plant Cell, Tissue Organ Culture 85, 271-283

Table 1. Transient GUS activity in immature embryos after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with or without centrifugation.

Pretreatment with centrifugation		GUS Activity Index	
		Variety	
Centrifugal acceleration (xg)	Time (second)	Yukihikari; experiment 1	Yukihikari; experiment 2
No centrifugation	—	5.8	4.6
1,000	1	17.8	13.0
1,000	10	16.2	13.2
1,000	60	13.2	14.9
20,000	1	38.0	59.2

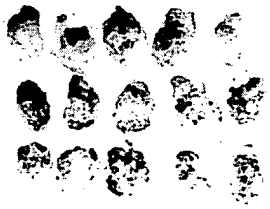


Control (not centrifuged)

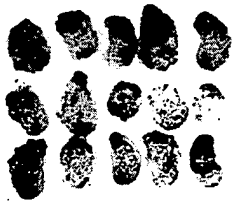


1,000 xg for 1 second,

1,000 xg for 10 seconds



1,000 xg for 60 seconds



20,000 xg for 1 second

Figure 1. Experiment 1; Histochemical GUS expression in immature embryos of Yukihikari after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with or without centrifugation.

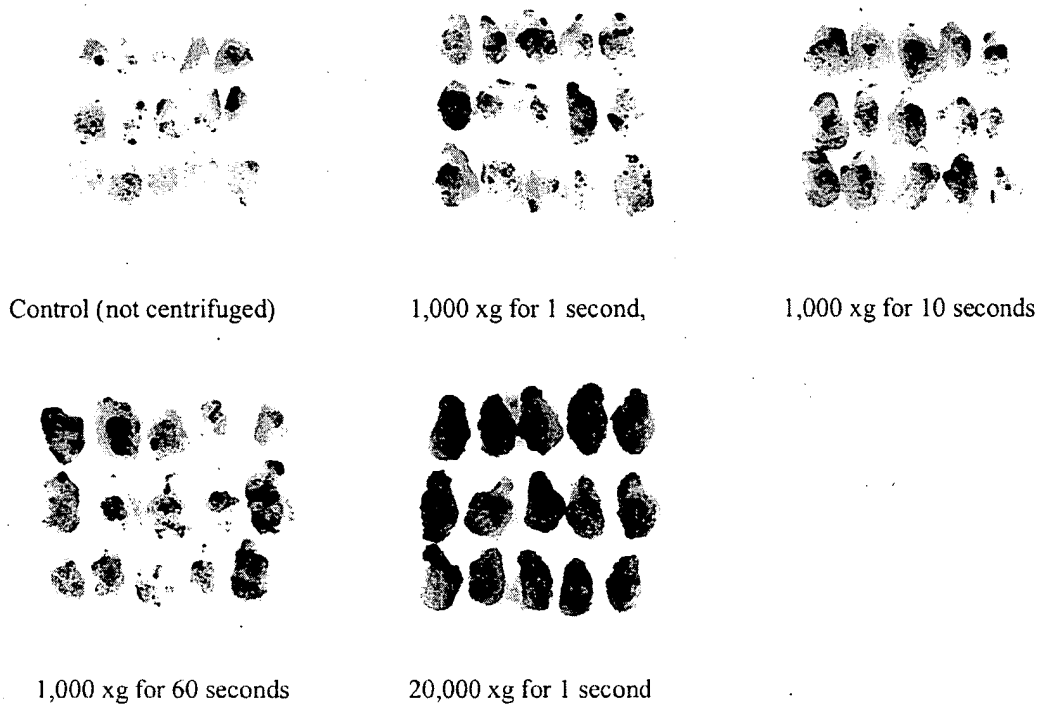


Figure 2. Experiment 2; Histochemical GUS expression in immature embryos of Yukihikari after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with or without centrifugation.

EXPERIMENT 2

Efficiency of *Agrobacterium*-mediated gene transfer in rice immature embryos pre-treated with various temperatures and the durations.

Materials and Methods

(1) *Agrobacterium* Strain and Plasmid

As the *Agro*bacterium and its vector, LBA4404(pTOK233) (Hiei et al. 1994) was used. The T-DNA region of pTOK233 has a hygromycin-resistant gene (*hpt*) regulated by the 35S promoter of CaMV and a GUS gene regulated by the 35S promoter of CaMV

and having the first intron of the catalase gene of castor-oil plant.

(2) Sample Varieties and Tissue

As the sample variety, IR64, which is the variety of Indica rice, was used. As the sample tissue, immature embryo was used. The preparation method of the tissue is the same as that described in the specification of the present patent application.

(3) Heat Treatment

Rice immature embryos were placed in a 1.5 ml centrifugal tube containing 1 ml of sterilized water. The tubes containing immature embryos were incubated in a water bath at various temperatures (34, 37, 40, 43, 46 and 49 °C) before infection with *A. tumefaciens* strain. The duration of the treatment was 3, 5, 10, 20, 40, 60, 100, 120 or 180 min for the immature embryos of rice. After the heat-treatment, the tubes were cooled in a water bath at 25 °C for 1min. In addition to the experimental plots, an experimental plot with no heat-treatment (room temperature, around 25°C) was added. In each experimental plot, 20 immature embryos were used. After the centrifugation, the immature embryos were infected with *Agrobacterium*.

(4) Infection of *Agrobacterium* and Co-culturing

The method of infection of the immature embryos with *Agrobacterium*, the method of co-culturing and the method of GUS assay of the immature embryos after the co-culturing were the same as described in specification of the present patent application. The co-culturing was carried out for 7 days. In the present test, the GUS expression levels in the immature embryos were expressed in values as GUS Activity Index as follows: Each of the immature embryos was then visually examined for the percentage of the sum of the blue areas to the total surface area of the scutellum. A score was given according to the percentage; score 0.0 was given for 0%, score 0.5 for between 0% and 1%, score 5.5 for between 1% and 10%, score 17.5 for between 10% and 25%, score 37.5 for between 25% and 50%, score 62.5 for between 50% and 75%, and score 87.5 for 75% and 100%. The average of the scores in an experimental plot was recorded as the GUS

Activity Index. The percentage (the GUS Activity Index) was then divided by the percentage for the untreated control plot to give a relative value.

Results and Discussion

A considerable enhancement of GUS expression in the immature embryos by the pre-treatment with heat was observed after the co-cultivation. In rice Indica IR64, the general tendency was that the higher the temperature of the treatment, the shorter was the time for the level of GUS expression to reach the peaks, which were detected after 120 min of treatment at 37 °C, 60 min at 40 °C, 40 min at 43 °C and 3 min at 49 °C (Figure 3). The height of the peaks did not differ much among the treatments (Figure 3). In each temperature prolonged incubation over the peak point increased the number of damaged embryos, which did not grow further on co-cultivation media or the next media for callus induction.

Cited Reference

Hiei Y, Ohta S, Komari T & Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271-282

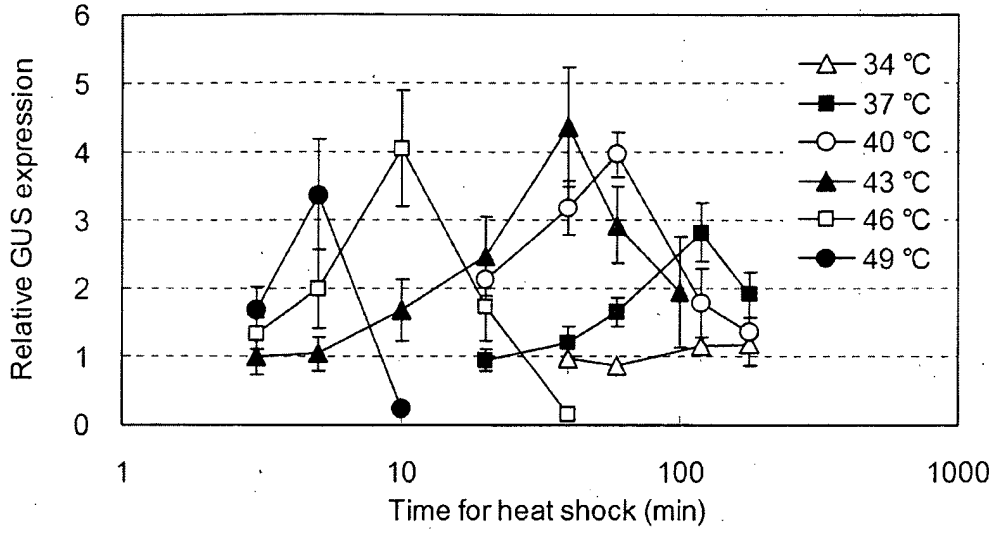


Figure 3 Relative GUS expression in immature embryos of rice variety IR64 pre-treated with heat.

Immature embryos of IR64 were treated with heat at given temperatures for defined lengths of time. The embryos were then inoculated with *A. tumefaciens* LBA4404(pTOK233). GUS in the immature embryos after the completion of co-cultivation was assayed histochemically with X-gluc. The GUS expression relative to that in untreated control is plotted with the range of standard error. Twenty embryos were used in each plot.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 6th day of October, 2008

Yukoh Hiei

Yukoh HIEI